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A single-tube multiplex reverse transcription–polymerase chain reaction for detection and differentiation of vesicular stomatitis Indiana 1 and New Jersey viruses in insects

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Abstract. A multiplex single-tube reverse transcription–polymerase chain reaction (RT-PCR) has been developed for the detection and differentiation of vesicular stomatitis viruses (VSV), Indiana 1 and New Jersey, from insect samples. Using this assay, detection of either or both viruses in as little as 20 fg of total RNA from tissue culture was achieved, along with detection of vesicular stomatitis (VS) RNA from macerates containing 2 infected mosquitoes in pools of 10–30 noninfected mosquitoes. Vesicular stomatitis virus was detected by RT-PCR in all culture-positive samples, and detection as low as 4 plaque forming units per milliliter was achieved. Comparison between RT-PCR and tissue culture revealed that RT-PCR was able to detect VSV in a volume of insect macerate averaging almost 100 times less than that required for detection by tissue culture. The reported RT-PCR is a potential valuable tool for rapid and sensitive detection and differentiation of VS in insects because intense work associated with viral isolation, the cytotoxicity of insect extracts, and separate virus identification steps can be avoided. Potential application to detection and differentiation of VSV serotypes from vertebrate hosts is addressed.

Vesicular stomatitis virus (VSV) causes vesicular lesions on the epithelia of the tongue and mouth, as well as coronary bands of hooves of cattle, pigs, and horses.[9] Humans, rodents, and numerous other mammals and fowl can also be infected.[12,16] Vesicular stomatitis virus has been isolated on many occasions in several species of insects, including mosquitoes, during viral epidemics and in forested endemic foci.[2,21–23] At least 2 groups of insects, sand flies (Lutzomyia spp) and black flies (Diptera: Simuliidae), have been shown to carry the virus in nature and to replicate and transmit VSV to susceptible hosts after laboratory infection.[2,13,20] In addition, it has been shown that black flies can act as vectors to disseminate VSV by cofeeding on a nonviremic host.[14] However, the exact role played by insects in VSV's natural cycle has not been definitely proven. Vesicular stomatitis (VS) is of considerable economic importance as quarantines must be enforced until diagnosis is complete because its clinical symptoms are indistinguishable from those of foot and mouth disease (FMD).[19] Diagnostic delays and inherent problems associated with herd quarantines result in underreporting by many producers, thereby increasing the risk of an actual FMD outbreak going unreported until it is too late for containment. Progress in epidemiological studies is necessary to understand the life cycle of VSV, which remains obscure.

Two major serotypes, New Jersey (NJ) and Indiana (IN), have been defined on the basis of cross-neutralization properties,[12] complement fixation, and genetic and biochemical relatedness.[7,12] Currently, insect studies use virus isolation techniques, which are time consuming, subject to cytotoxicity, and require virus identification after isolation. Reverse transcription–polymerase chain reactions (RT-PCR) have been reported for VSV but are not routinely performed in diagnostic laboratories.[17,19] The usefulness of the VSV RT-PCRs previously reported is limited in that only one of the major VSV types (NJ or IN) can be detected in each reaction,[19] requiring the performance of 2 separate RT-PCR reactions per sample, or the size differentiation of the products is not easily discernable on agarose gel electrophoresis.[17] Diagnostic sensitivity of these PCRs has not been reported.

The objectives of this study were to develop and experimentally evaluate a single-tube one-step multiplex RT-PCR for the detection and differentiation of VSV-IN and VSV-NJ in insect macerates and to determine the potential application of the assay to vertebrate host samples. This RT-PCR provides advantages in terms of time and reagent costs when compared with current methods of detection by tissue culture and

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typing. Furthermore, it can be combined with virus isolation in cell culture for rapid typing of cytopathic-positive samples. Epidemiological studies conducted during outbreaks would be greatly enhanced by use of this RT-PCR. The potential application of this assay to epithelial tissues from animals exhibiting clinical signs of vesicular disease would facilitate rapid diagnosis and reporting of results.

Materials and methods

Viruses. Reference strains of VSV-IN1 (IN1) and NJ-Hazelhurst were kindly provided by Dr. Charles Calisher of the Arthropod Borne Infectious Disease Laboratory at the Colorado State University. Five clinical equine isolates of VS-IN1 and VS-NJ viruses originating from outbreaks in the southwestern United States were obtained from Dr. Sabrina Swenson, Head, Bovine and Porcine Viruses Section, National Veterinary Services Laboratory, USDA, Animal and Plant Health Inspection Service, Ames, Iowa.

Cell culture and infection. Viral stocks were grown on BHK-21[a] cells by infecting cell monolayers in 75-cm^2 flasks with 10^8 tissue culture infectious dose 50 ($TCID_{50}$) (100 μl) of each VSV stock and incubating for 24 hr or until approximately 70% cytopathic effect was observed. Cell supernatant containing the VSV was clarified by centrifugation at 100 × *g* for 3 min at room temperature, aliquoted, and frozen at −70 C. Noninfected cultures were incubated, clarified, and stored at the same time for use as negative controls.

Mosquito infection. To compare and to validate detection of VSV in insects by tissue culture plaque assay and by RT-PCR, mosquitoes were chosen as an insect model. Vesicular stomatitis virus has previously been isolated from mosquitoes,[15] and the size of mosquitoes facilitated the thoracic injection of the virus. Mosquitoes (*Aedes triseriatus*) were injected intrathoracically with 0.6 μl of IN or NJ culture supernatant containing 2.9×10^8 plaque forming units (PFU)/ml or 1.9×10^8 PFU/ml, respectively. Intrathoracic injections of similar levels of virus have been reported.[6,8] Uninfected mosquitoes were stored immediately at −70 C for use as negative controls. Infected mosquitoes were maintained in an insectary at 20–23 C and 70% relative humidity. Mosquitoes were allowed to feed on sugar cubes and water. Groups of 15 mosquitoes were harvested by storing at −70 C on days 0, 1, 3, 5, 7, 9, 11, and 13 after infection.

Mosquito pools. A sample of insects collected from a field study would potentially contain many noninfected insects along with a few infected insects. Although the current study did not involve field-collected insects, sample sets were devised to more closely resemble those that may be obtained from the field in future studies. This limited comparison involved the addition of 10–30 noninfected mosquitos to selected subsets of infected mosquitos, which were then processed together for determination of PFU per milliliter and for detection by RT-PCR. Subsets investigated in this manner included VS-IN from days 5 and 13 after infection and VS-NJ from days 0 and 7 after infection.

RNA extraction and purification. Two whole mosquitoes, representing a subset of 1 day's harvest, maintained at −70 C were removed and placed in 300 μl cold viral dilution media containing minimum essential medium (MEM) without fetal bovine serum. Additional supplements used when grinding mosquitoes included double antibiotics/antimycotics and 1 U/μl RNase inhibitor.[b] The mosquitoes were ground over ice with plastic pestles in 1.7 ml microcentrifuge tubes . Debris were pelleted by centrifugation at 12,000 × *g* for 1 min. A 25-μl aliquot of clarified supernatant was removed and diluted in viral dilution medium, on ice, for the plaque assay described below. A 250-μl aliquot from the same sample of clarified mosquito supernatant was removed and added immediately to 750 μl of triphasic purification reagent,[c] mixed, and after incubation on ice for 5 min, stored at −70 C until extraction. For the extraction, samples of infected cell culture supernatant or clarified mosquito supernatant were thawed rapidly at 37 C and placed immediately on ice. Ribonucleic acid was extracted by the acid guanidine method as per manufacturer's instructions.[c] Purified RNA was solubilized in 50 μl of water containing 1 U of RNase inhibitor/μl,[d] and quantified by fluorimetry with the use of fluorescent dye.[e] Aliquots were kept frozen at −70 C.

Although a volume of macerate 10 times that used for plaque assay was used to prepare lysate for the RT-PCR, the actual final volume of macerate used for each RT-PCR reaction was 2 μl of a 5 times concentration, or 10 μl.

Plaque assay. Viruses were quantified by plaque assay as described previously.[18] Briefly, culture supernatant or clarified mosquito supernatants were serially diluted in MEM, and BHK-21 monolayers in 6-well plates were inoculated with 0.5 ml of each dilution, in duplicate. Plaques were observed after 38–44 hr, following staining with 0.5% neutral red for 2 hr. Wells containing 4.8–48 plaques were considered countable. A negative control was included on each plate, consisting of the dilution medium without virus, uninfected culture supernatant, uninfected mosquito-clarified supernatant, or phosphate-buffered saline. After confirming absence of plaques in the negative controls, PFU per milliliter were calculated by taking the reciprocal of the countable dilution average and multiplying the same by 2. Two independent titrations were performed on each insect subset and the results averaged.

RT-PCR. Oligonucleotide primers were chosen that targeted portions of the P (polymerase-associated phosphoprotein) gene of IN[19] and the L (large polymerase protein) gene of NJ-subtype Hazelhurst.[4,17]

These targets were chosen based on the viral strains provided, the ease of amplification, specificity, and size differentiation of products. Different gene targets were also chosen to decrease the possibility of interactions between primer sets, which have been observed previously (Luis L. Rodriguez, unpublished data). Specificity of the primers has been determined previously.[17,19] The reverse IN primer was modified to contain the bases C and A, as per the GenBank sequence, in place of Y and R, as published.[19] The forward NJ primer was modified by 1 base to conform to the Hazelhurst subtype. The forward primer sequence of IN, beginning at position 179 of the P gene, is 5′-GCA-GAT-GAT-TCT-GAC-AC-3′. The reverse primer for IN is located at position 793, and the sequence is 5′-GAC-TCT-CGC-CTG-ATT-GTA-3′. The primers for NJ are located at positions

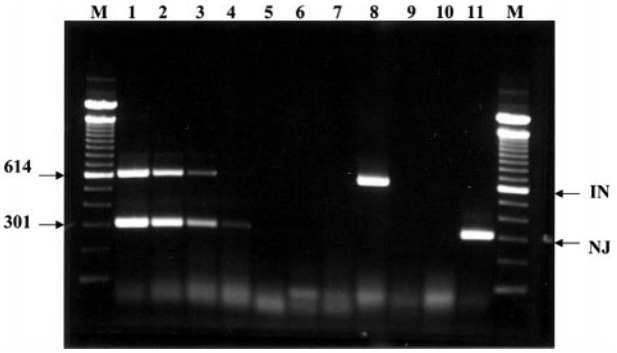

Figure 1. Detectability and specificity of the RT-PCR using total RNA from tissue culture. Agarose gel (1.8%) stained in ethidium bromide. Lane M: 100-bp marker; lane 1: 2,000 fg IN and NJ; lane 2: 200 fg IN and NJ; lane 3: 20 fg IN and NJ; lane 4: 2.0 fg IN and NJ; lane 5: 2,000 fg IN and NJ, no reverse transcriptase; lane 6: total RNA from uninfected tissue culture; lane 7: negative control (water as template); lane 8: IN RNA with IN primers; lane 9: IN RNA with NJ primers; lane 10: NJ RNA with IN primers; lane 11: NJ RNA with NJ primers.

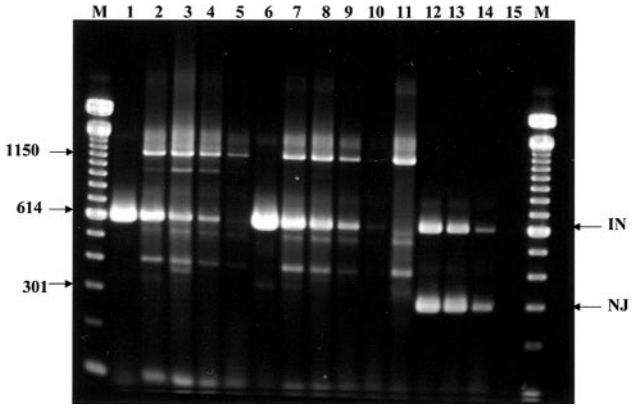

Figure 2. Reverse transcription–polymerase chain reaction amplification and end point dilution of VS-IN1 RNA from 2 infected mosquitoes in pools of 10–30 noninfected mosquitoes. Agarose gel (1.8%) stained in ethidium bromide. Lane M: 100-bp markers; lane 1: IN day 5 pool RNA, undiluted; lane 2: IN day 5 pool RNA, 1:100; lane 3: IN day 5 pool RNA, 1:1,000; lane 4: IN day 5 pool RNA, 1:10,000; lane 5: IN day 5 pool RNA, 1:100,000; lane 6: IN day 13 pool RNA, undiluted; lane 7: IN day 13 pool RNA, 1:100; lane 8: IN day 13 pool RNA, 1:1,000; lane 9: IN day 13 pool RNA, 1:10,000; lane 10: IN day 13 pool RNA, 1:100,000; lane 11: uninfected mosquito RNA as template, 1:100; lane 12: positive control RNA from infected tissue culture, 2,000 fg total IN and NJ RNA; lane 13: positive control RNA from infected tissue culture, 200 fg total IN and NJ RNA; lane 14: positive control RNA from infected tissue culture, 20 fg total IN and NJ RNA; lane 15: negative control (water as template in PCR).

313 and 613, and the sequences are 5′-TTG-GTT-CGG-AAC-TTG-GAT-TC-3′ and 5′-ACT-CAT-GCG-GTA-TTT-ACC-CTT-G-3′, respectively.

Final concentrations in each 25 μl reaction were 50 mM KCl, 10 mM Tris–HCl, pH 9.0, 0.1% Triton X-100, 2.0 mM $MgCl_2$, 0.3 mM each deoxynucleotide triphosphate (dNTP),[d] 5% dimethyl sulfoxide, 1.2 μM each IN primer,[f] 0.8 μM each NJ primer,[f] 3 U of Avian Myeloblastosis virus (AMV) reverse transcriptase,[g] 2 U of *Taq* polymerase,[h] and 20 U of RNase inhibitor.[d] All reagents were combined in a master mix or cocktail, which was then aliquoted to individual 0.5 ml reaction tubes, 2 μl of RNA was added, and reactions overlaid with 50 μl of wax.[i] Transcription and amplification were carried out with the following thermal cycling[i] program: 50 min at 46 C for reverse transcription, 3 min at 94 C for initial denaturation and inactivation of the reverse transcriptase, followed by 40 cycles of 1 min at 94 C, 2 min at 50 C, and 3 min at 72 C. Reactions were held at 4 C until the products could be analyzed by gel electrophoresis. Products were mixed with tracking dye and separated by electrophoresis for 10–20 min in 1.8% agarose gel and Tris-Acetate EDTA (TAE) buffer at a constant 275 V.[j] Photo documentation followed UV visualization and staining with ethidium bromide.

Negative controls consisted of RNA purified from uninfected culture supernatant, RNA purified from uninfected mosquitoes, or water in place of RNA in the PCR. To ensure that RNA was being transcribed and amplified, a DNA control was included, which consisted of all reagents and template, but the reverse transcriptase was eliminated or inactivated. To prevent carryover contamination and RNase contamination, all reactions were set up in a dedicated RT-PCR hood with dedicated decontaminated pipettors and in a room separate from that used for gel electrophoresis of amplified products. Barrier tips and nuclease-free supplies and reagents were used at all times. Previously quantified RNA was thawed, diluted to 1,000,100, and 10 fg/μl was used to confirm the sensitivity of the RT-PCR each time an RT-PCR was conducted. Dilutions were performed in nuclease-free water containing RNase inhibitor. Two microliters of template was added to each PCR reaction as noted previously. Total RNA purified from mosquitoes was initially tested by RT-PCR at a 1:10 dilution for confirmation of amplification, and then 1:100, 1:1,000, 1:10,000, 1:100,000, 1:250,000, 1:500,000, and 1:1,000,000 dilutions were tested to determine the titer. Values were averaged between the 2 independent titrations.

Sizes of the products were determined by comparison with 100-bp markers.[h] A 614-bp band indicated amplification of the appropriate P gene portion of VS-IN1, and a 301-bp band indicated amplification of the L gene portion of VS-NJ. Polymerase chain reaction products were purified by use of columns[k] and sent to the University of California, Davis, California, sequencing facility. Sequences obtained were compared with published sequences using the BLAST program.[l]

Primer and gene sequences were obtained from GenBank. The accession numbers are X04453 for the phosphoprotein (P) of IN1 and M20166 for the L gene of NJ subtype Hazelhurst.

Results

The RT-PCR reported in this study consistently detected VSV-IN1 or VS-NJ RNA, or both, in as little as 20 fg of total RNA from tissue culture (Figs. 1–5) and in as much as 430.2 ng of total RNA from a mosquito subset extraction (Fig. 2, lane l). No amplifica-

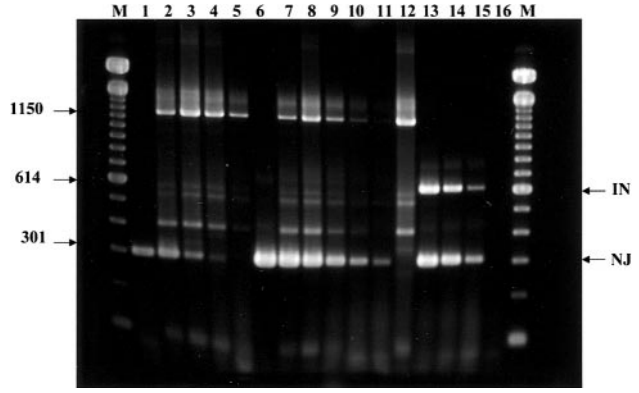

Figure 3. Reverse transcription–polymerase chain reaction amplification and end point dilution of VS-NJ RNA from 2 infected mosquitoes in pools of 10–30 noninfected mosquitoes. Agarose gel (1.8%) stained with ethidium bromide. Lane 1: NJ day 0 pool RNA, undiluted; lane 2: NJ day 0 pool RNA, 1:100; lane 3: NJ day 0 pool RNA, 1:1,000; lane 4: NJ day 0 pool RNA, 1:10,000; lane 5: NJ day 0 pool RNA, 1:100,000; lane 6: NJ day 7 pool RNA, undiluted; lane 7: NJ day 7 pool RNA, 1:100; lane 8: NJ day 7 pool RNA, 1:1,000; lane 9: NJ day 7 pool RNA, 1:10,000; lane 10: NJ day 7 pool RNA, 1:100,000; lane 11: NJ day 7 pool RNA, 1:1,000,000; lane 12: uninfected mosquito RNA as template, 1:100; lane 13: positive control RNA from infected tissue culture, 2,000 fg total IN and NJ RNA; lane 14: positive control RNA from infected tissue culture, 200 fg total IN and NJ RNA; lane 15: positive control RNA from infected tissue culture, 20 fg total IN and NJ RNA; lane 16: negative control (water as template in PCR).

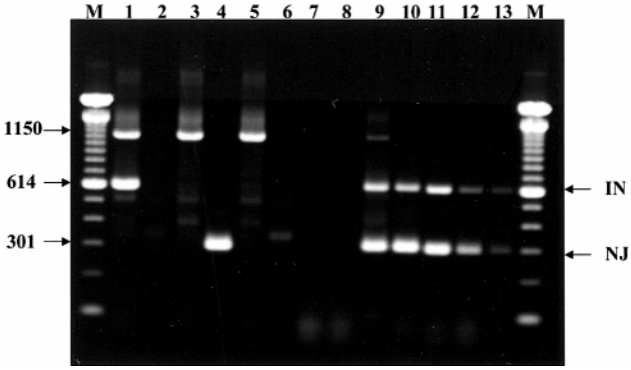

Figure 4. Specificity of RT-PCR using RNA extracts from infected mosquitoes in pools of noninfected mosquitoes and removal of nonspecific products with DNAse treatment. Agarose gel (1.8%) stained with ethidium bromide. Products in lanes 1, 3, and 5 amplified using IN primer set only. Products in lanes 2, 4, and 6, amplified with NJ primer set only. Products in lanes 7–13 amplified with both primer sets. Lane M: 100-bp markers; lanes 1 and 2: RNA from subset of IN-infected mosquitoes in pool of noninfected mosquitoes; lanes 3 and 4: RNA from subset of NJ-infected mosquitoes in pool of noninfected mosquitoes; lanes 5 and 6: uninfected mosquito RNA, 1:100; lane 7: negative control (water as template); lane 8: IN and NJ-infected mosquito RNA, pretreated with RNAse; lane 9: IN and NJ-infected mosquito RNA, no nuclease pretreatment; lane 10: IN and NJ-infected mosquito RNA, pretreated with DNAse; lane 11: positive control RNA from infected tissue culture, 2,000 fg total IN and NJ RNA; lane 12: positive control RNA from infected tissue culture, 200 fg total IN and NJ RNA; lane 13: positive control RNA from infected tissue culture, 20 fg total IN and NJ RNA.

tion was detected in the negative controls, and no cross-reactions were observed between IN templates and NJ primers, and vice versa, when using RNA purified from infected tissue cultures (Fig. 1).

For each subset of infected mosquitoes, the PFU per milliliter and RT-PCR titer (defined as the reciprocal of the last positive dilution) were determined, and the lower limit of PFU per reaction was calculated and used to establish the minimum PFU that could be detected per milliliter of mosquito macerate (Table 1). Vesicular stomatitis virus RNA was detected at each time point, and less than 1 PFU/reaction by RT-PCR was consistently detected from day 1 through day 13 after infection. The effective volume of insect macerate used for each of the 2 assays was divided by the reciprocal of the countable to compare the detection level achievable by plaque assay with the corresponding value achievable by RT-PCR. As shown in Table 2, the absolute volume of macerate required to detect the virus by RT-PCR was considerably lower than that required to detect the virus by plaque assay. The average volume of macerate required to detect a positive by RT-PCR was 5.6% of that required by plaque assay and ranged from 0.3% to 12.5%. The volume of macerate required to detect a positive by RT-PCR did increase when the pools of noninfected mosquitoes were added to the infected mosquitoes but remained at a

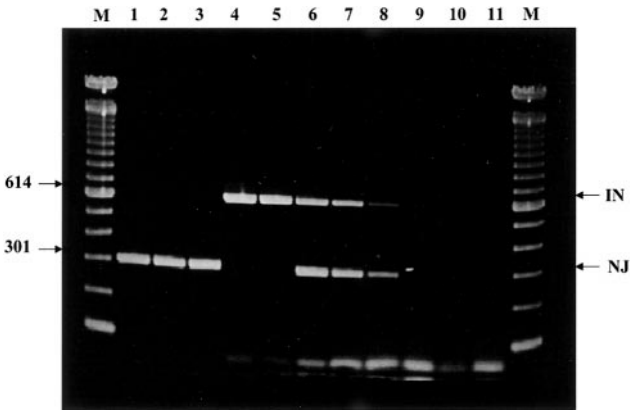

Figure 5. Reverse transcription–polymerase chain reaction amplification of RNA from clinical VSV isolates grown in tissue culture. Agarose gel (1.8%) stained in ethidium bromide. Lane M: 100 bp markers; lane 1: NJ isolate 95-29267; lane 2: NJ isolate 95-44278; lane 3: NJ isolate 95-35864; lane 4: IN isolate 97-33486; lane 5: IN isolate 98-3648; lane 6: positive control, 2,000 fg each IN and NJ; lane 7: positive control, 200 fg each IN and NJ; lane 8: positive control, 20 fg each IN and NJ; lane 9: negative control, uninfected cell culture supernatant; lane 10: negative control for DNA (no reverse transcriptase); lane 11: negative PCR control (water as template).

Table 1. Plaque assay counts and lowest detection limits by RT-PCR of samples from infected mosquitoes, per day after infection.

Day after infection	Plaque assay		RT-PCR		
	PFU/ml	PFU/μl	Last dilution positive $\times 10^{-1}$	Lower limit of PFU detected per reaction*	Minimum PFU detected per ml
VS-INI					
0	870,000	870	550	15.82	7,909
1	33,500	335	550	0.609	305
3	1,425,000	1,425	375,000	0.038	19
5	1,200,000	1,200	375,000	0.032	16
7	475,000	475	375,000	0.013	6
9	340,000	340	175,000	0.019	10
11	185,000	185	250,000	0.007	4
13	335,000	335	250,000	0.013	7
5†	180,000	180	10,000	0.180	90
13†	230,000	230	100,000	0.023	12
VS-NJ					
0	960,000	960	10,000	0.96	480
1	265,000	265	10,000	0.27	133
3	4,850,000	4,850	550,000	0.09	44
5	2,600,000	2,600	300,000	0.09	45
7	2,150,000	2,150	750,000	0.03	14
9	2,950,000	2,950	750,000	0.04	19
11	3,500,000	3,500	625,000	0.06	28
13	1,850,000	1,850	750,000	0.02	12
0†	1,600,000	1,600	10,000	1.60	800
7†	540,000	540	500,000	0.01	6

* Calculation: Plaque forming unit/μl × 5 (concentration factor during purification) × 2 μl used per RT-PCR reaction/last dilution positive by RT-PCR. Example: For VSIV 3 days after infection, tissue culture detected 1,425,000 PFU/ml or 1,425 PFU/μl. 1,425 PFU/μl × 5 × concentration × 2 μl/375,000 = 0.038 PFU detected in the RT-PCR reaction.

† Samples of 2 infected mosquitoes from day indicated in pools of 10–30 noninfected mosquitoes.

level substantially lower than that required by plaque assay.

The identities of the PCR products obtained by RT-PCR for both VS-INI and VS-NJ were confirmed by sequencing to be those of the viruses inoculated.

VS RNA was detectable and identifiable by use of the multiplex RT-PCR in macerates from the infected mosquitoes as well as from macerates of sets of mosquitoes that contained two infected mosquitoes pooled with 10–30 noninfected mosquitoes (Figs. 2, 3). The lower limit of detection per RT-PCR reaction, although slightly higher in most cases than the corresponding set processed without additional uninfected mosquitoes, averaged below 1 PFU (Table 1). No viral plaques or RT-PCR products were obtained when uninfected mosquitoes were processed alone (Figs. 2, 3).

A nonspecific amplification product of about 1,150 bp was observed when diluted nucleic acid from insects was used as template (Figs. 2, 3). Treatment of the extracted RNA with DNase^{m} before amplification resulted in disappearance of the nonspecific amplicons (Fig. 4).

In addition to detecting VSV in infected insects, the ability of this multiplex RT-PCR to rapidly identify the

Table 2. Comparitive detection of VSV from (A) infected mosquitoes and (B) infected mosquitoes in pools of noninfected mosquitoes by tissue culture and by RT-PCR.

Tissue culture	A		B	
	IN	NJ	IN	NJ
Actual volume (μl) of macerate used	25	25	25	25
Divided by the reciprocal of average dilution detectable	2×10^3	3×10^4	1×10^4	5×10^4
Volume of macerate (μl) needed for detection $\times 10^{-5}$	1,300	83	250	50
RT-PCR				
Actual volume (μl) of macerate used (2 μl of a 5× concentration)	10	10	10	10
Divided by the reciprocal of average dilution detectable	2.25×10^5	4.68×10^5	5.5×10^4	2.6×10^5
Volume of macerate (μl) needed for detection $\times 10^{-5}$	4	2	18	4

VSV serotype in clinical isolates grown in tissue culture was assessed. When RNA extracts from 3 VS-NJ and 2 VS-INI field isolates were tested, the expected size product was amplified (Fig. 5).

Discussion

The developed RT-PCR could detect as little as 4 VSV PFU/ml from infected mosquitoes (Table 1). This detection limit is superior or comparable to reported detection limits of similar assays.[1,3,5,10,24] The significant increase in the level of virus assayed by tissue culture after day 1 postinfection indicates that the virus replicated in the mosquitoes, as noted by other researchers.[13] The ability to detect VS RNA by this multiplex RT-PCR in as little as 20 fg or in as much as 430.2 ng of insect total RNA demonstrates the wide range of acceptable template concentrations that may be used. Negative controls confirmed that only RNA was transcribed and amplified, no virus had contaminated the control cultures, and no contaminating RNA or DNA was contained in any of the reagents used in this study.

The minimum achieved by RT-PCR should not be used to compare with the actual achieved by the other method (tissue culture plaque assay) because each method has a different approach to detect the viral particles. Thus, a comparison method using insect macerate material was used. This comparison indicated the superiority of RT-PCR in detection of viral particles over the tissue culture plaque assay. Although both methods detected all the known positives, the volume of mosquito macerate necessary to achieve detection differed significantly between the 2 methods. The RT-PCR required less macerate, i.e., less virus in order for positive detection to take place. Further analysis is necessary with a complete field trial to determine whether the results and assay are applicable to a sample of larger size. The volume of macerate required to detect a positive by RT-PCR increased when the pools of noninfected mosquitoes were added to the infected ones; however, the volume of macerate required by RT-PCR to detect a positive remained significantly lower than the volume required by tissue culture.

The multiplex RT-PCR is slightly asymmetric because it can detect VS-NJ RNA in as little as 2.0 fg of total RNA, but VS-IN can be detected only when 20 fg or more of total RNA is used as template (Fig. 1). This effect is somewhat mitigated by using the IN and NJ primers at final concentrations of 1.2 and 0.8 μm, respectively. Further investigation of the primers with the BLAST and Oligo 5.0 programs revealed a 4-nt hairpin in the INP-S primer. This may explain why the IN reaction is not as robust as the NJ reaction. Additional primers were tested, but they either performed poorly or did not perform at all (data not shown).

Although the primers are specific for their respective VSV genes and do not cross-react with each other, nonspecific RT-PCR amplicons occur when mosquito nucleic acid is used as the template. The nonspecific RT-PCR band at about 1,150 bp does not pose a problem when determining presence of VS RNA because the size is easily distinguishable from that of IN or NJ. Moreover, treatment of the extract with DNase before amplification removed nonspecific products from the reactions, resulting in no impact to the sensitivity or specificity of the RT-PCR. Ribonucleic acid extracts can be treated with DNase to ensure that there is no nucleic acid competing for available primers and thus possibly masking low-level positives.

Amplification of RNA from VS-NJ and VS-IN1 clinical isolates was successful with the multiplex RT-PCR. Although one of the clinical isolates was the Ogden strain,[11] and the primers are for the Hazelhurst strain, it is not surprising that amplification occurred because the primer sequences for the L gene of Ogden and Hazelhurst strains differ by 1 base on the forward primer only. This indicates that the RT-PCR is not limited to the Hazelhurst strain and is applicable to both strains of VS-NJ. These clinical isolates were included in this study to confirm that virus isolated from clinical samples by another laboratory were capable of being amplified and identified correctly by the RT-PCR described in this study and as such were not intended to replace further study using a statistically significant number of field samples.

This RT-PCR may be used to detect VSV RNA in infected insects with a detection level surpassing that of isolation, with the additional advantage that it will identify the virus as VSV and its serotype. This rapid RT-PCR can be used to identify the virus causing cytopathic effects (CPE) in cells either from clinical cases or from insects being tested by virus isolation. This method is faster than complement fixation or neutralization, which are the tests currently used for virus identification.

The multiplex RT-PCR developed and optimized is rapid, sensitive, and reliable. Problems associated with conventional virus isolation such as the cytotoxic effects of insect macerates can be avoided by use of the RT-PCR, and time-consuming virus identification assays would not be necessary.

Either or both major types of VS, IN1, and NJ can be detected and differentiated in a single reaction, with results available in as little as 1 day. Implementing the RT-PCR in the research laboratory should enable scientists to make progress in understanding the life cycle of VSV in its natural setting. Future research endeav-

ors will include testing of field-collected insects and mammal tissues for further validation of this assay.

Acknowledgement

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Sources and manufacturers

a. BHK-21; American Type Culture Collection, Manassas, VA.
b. SuperaseIN RNase inhibitor; Ambion, Austin, TX.
c. TRI-REAGENT, and TRI-LS; Molecular Research Center, Inc., Cincinnati, OH.
d. Roche Molecular Biochemicals, Indianapolis, IN.
e. Ribogreen fluorescent dye; Molecular Probes, Eugene, OR.
f. Operon, Alameda, CA.
g. Promega Corporation, Madison, WI.
h. GIBCO-BRL/Invitrogen, Rockville, MD.
i. Chill-Out wax, PTC-100 thermalcycler; MJ Research, Waltham, MA.
j. RAGE 100× electrophoresis system; Midwest Scientific, Valley Park, MS.
k. QIAquick columns; QIAgen, Valencia, CA.
l. National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD.
m. DNA-free Ambion, Austin, TX.

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